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Exploring the mechanism of lipid transfer during biosynthesis of the acidic lipopeptide antibiotic CDA

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ABSTRACT

The non-ribosomally synthesized lipodepsipeptide CDA belongs to the group of acidic lipopeptide antibiotics, whose members feature a fatty acid side chain that strongly affects their antimicrobial activity. This study elucidates the *N*-acylation of the N-terminal serine in the CDA peptide chain. This reaction is referred to as lipoinitiation and is shown to be catalyzed by the dissected starter C domain found at the N-terminus of Cda-PSI. The recombinantly produced C domain specifically interacts with 2,3-epoxyhexanoyl-*S*-ACP and catalyzes the transfer of the fatty acid moiety onto the amino group of PCP-bound serine with high selectivity for both carrier protein bound substrates at the donor and acceptor site.

1. Introduction

Acidic lipopeptides constitute an important class of non-ribosomally synthesized peptide antibiotics. Daptomycin is probably the most prominent member of this group, due to its application as a last-resort antibiotic in cases of infections by multidrug resistant pathogens, beside other well known representatives like A54145 from *Streptomyces fradiae* or calcium-dependent antibiotics (CDA) produced by the model streptomycete *Streptomyces coelicolor* A3(2) [1]. Common features of this class are the fatty acid moiety N-terminally attached to the peptide chain, the conserved position of D-configured and achiral amino acids and the Ca^{2+}-binding motif DxDG [2], which is essential for antibiotic activity by neutralising the acidic peptide residues thereby improving the binding to bacterial cytosolic membranes [3]. The biosynthesis of the branched cyclic lipodepsipeptide CDA is accomplished by three NRPS enzymes (Cda-PS1-3) that comprise eleven modules associated with the peptide assembly and cyclization (Fig. 1A) [4]. The N-terminal lipid moiety facilitates the penetration of lipopeptides into the plasma membrane of Gram-positive bacteria and thus dramatically affects their antimicrobial activity [1,5,6]. Therefore, the nature of the fatty acid group is important when considering the generation of new more potent and less toxic antimicrobial agents. Recently, the mechanism of activation and tailoring processes involved in the formation of the β-amino fatty acid moiety of the NRP/PK hybrid mycosubtilin [7] as well as the activation and transfer of the β-hydroxy fatty acid found in surfactin have been elucidated [8]. The fatty acid moiety in CDA is a unique 2,3-epoxyhexanoic acid (Fig. 1B) which is invariant in contrast to all other known lipopeptides of this class where the fatty acid residues can vary in length or degree of saturation as well as branching. Within the biosynthetic gene cluster of CDA, a putative fatty acid biosynthesis (*fab*) cluster encoding five proteins proposed to be involved in the synthesis and modification of 2,3-epoxyhexanoic acid (Fig. 1C) was identified. The gene SCO3249 encodes an acyl carrier protein (ACP) similar to ACPs from fatty acid and polyketide synthases. The gene products FabF3 and FabH4 share the highest homology to β-ketoacyl-ACP synthase KAS-II and -III enzymes, respectively. These are proposed to be involved in the synthesis of hexanoyl-*S*-ACP (Fig. 1C). In contrast, HxcO and HcmO have been shown to be a FAD-dependent hexanoyl-ACP oxidase and a hexenoyl-ACP monooxygenase, respectively, and were shown in vitro to generate the epoxy group found in the fatty acid side chain of CDA [9].

In this study we focus on the transfer reaction of the fatty acid moiety (FA) onto the peptide chain of CDA which is shown to be catalyzed by the excised initiation C domain of the first NRPS

Abbreviations: ACP, acyl carrier protein; C domain, condensation domain; CDA, calcium-dependent antibiotic; CP, carrier protein; CoA, coenzyme A; FA, fatty acid; HPLC-ESI-MS, high performance liquid chromatography electrospray ionisation mass spectrometry; LB, lysogenic broth; NRPS, non-ribosomal peptide synthetase; NTA, nitrilotriacetic acid; PCP, peptidyl carrier protein; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gelelectrophoresis; SEC, size exclusion chromatography

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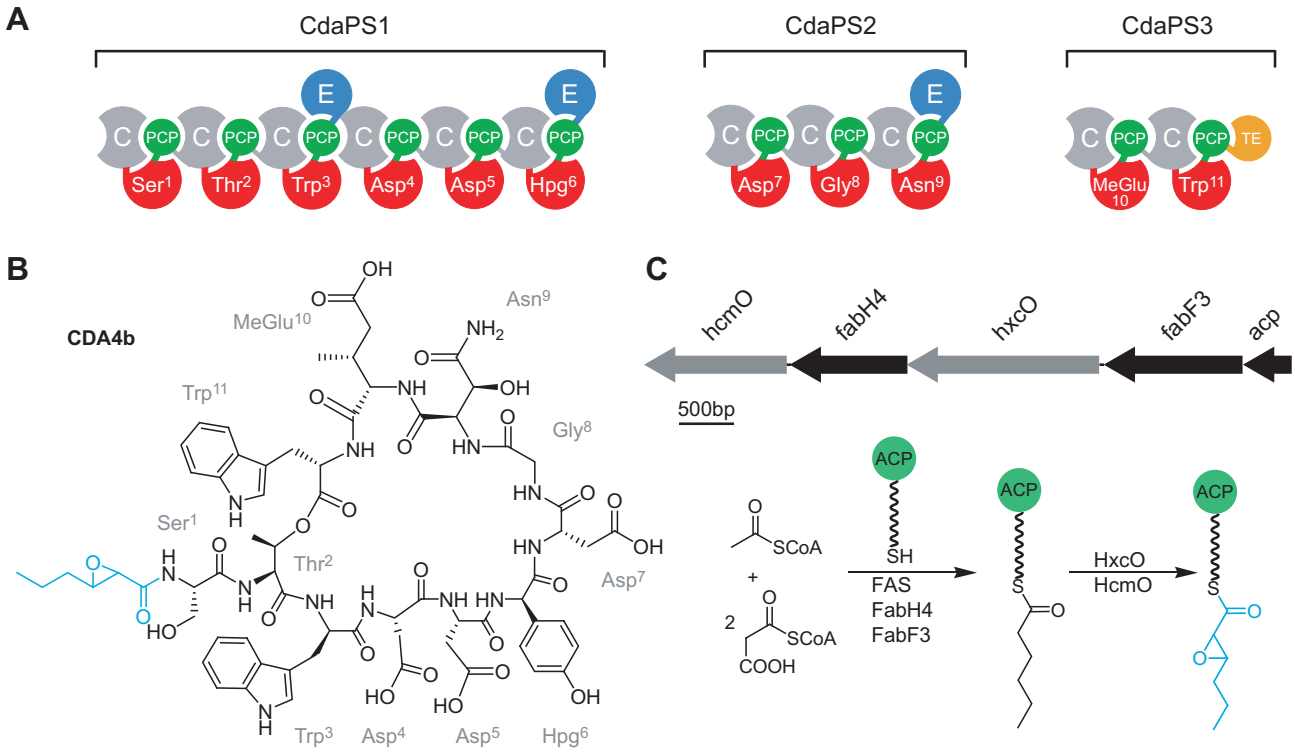

Fig. 1. Biosynthesis and structure of CDA. (A) Domain organization of the three CDA synthetases CdaPS1–3. C domains are shown in grey, A domains in red, PCP domains in green, E domains in blue and the TE domain in orange. Hpg: hydroxyphenyl-glycine; MeGlu: methyl-glutamate; (B) Structure of CDA4b produced by *Streptomyces coelicolor* A3(2), the fatty acid moiety is highlighted in blue; (C) The *fab* genes of the CDA biosynthetic gene cluster and the putative mechanism of fatty acid biosynthesis prior to incorporation into the peptide chain.

subunit Cda-PS1. The results obtained in this study enable new insights into the lipoinitiation reaction mechanism by identifying the carrier protein(CP)-bound substrates involved and defining the substrate specificity of the starter C domain.

2. Materials and methods

Materials and methods are described in the Supplementary data.

3. Results

3.1. Purification of proteins and assembly of FA-S-ACP and AA-S-PCP

Since this study aimed to investigate the initiation of CDA biosynthesis, which is the acylation of the N-terminal serine with 2,3-epoxyhexanoic acid, all proteins involved in this reaction were heterologously produced. The epoxidation enzymes HxcO and HcmO as well as the acyl carrier protein SCO3249 were expressed and purified as previously described [9]. Since heterologous expression of the entire initiation module of the CDA synthetase CdaPS1 produced unsoluble protein despite different attempts to increase solubility by altering domain borders and testing various expression conditions, we decided to excise and express the C domain (Cda-C1) as a singular protein. This approach was recently shown to generate a soluble and active initiation C domain in the surfactin system [8]. Cda-C1 was cloned into a pET28a(+) derived vector and expressed in *Escherichia coli* BL21(DE3). The subsequent purification by Ni^{2+}-NTA affinity chromatography yielded 2.6 mg/L culture of the C domain as a C-terminally His_6-tagged protein. The variants H156A and H157A of the C domain containing point mutations of the putative catalytic motif HHxxxDG were constructed by site-directed mutagenesis. The variant H157A could be obtained in a comparable yield to the parental protein of about 2.5 mg/L culture. In contrast, the variant H156A generated unsoluble protein under various expression conditions. Since the dedicated peptidyl carrier protein (PCP) domain from the initiation module of CdaPS1 could not be expressed as a recombinant protein, we used the homologous PCP domains of the surfactin synthetase SrfAA and of the daptomycin synthetase DptA. All proteins were identified by SDS–PAGE and subsequent mass spectrometry after tryptic digestion (Fig. S1).

To obtain the putative C domain substrates 2,3-epoxyhexanoyl-*S*-ACP and Ser-*S*-PCP, the corresponding chemically synthesized CoA thioesters were used with the promiscous 4′-phosphopantetheinyl transferase Sfp to convert the *apo*-carrier proteins to their active *holo*-forms (Fig. 2). Epoxidation of hexanoyl-ACP was accomplished by incubation with HxcO or HcmO and the cofactors FAD and NADH. To remove Sfp and the epoxidation enzyme, the modified carrier proteins were subjected to size exclusion chromatography (Fig. S2). To verify the proper loading state of CPs, samples were subjected to mass spectrometric analysis. In all cases, the correct loading state could be confirmed. Additionally, signals of *holo*-CPs were observed, resulting from substrate release through thioester hydrolysis (Fig. S3).

3.2. Condensation reaction catalyzed by Cda-C1

To investigate the catalytic activity of the dissected C domain Cda-C1, assays containing the purified C domain Cda-C1 and the substrates 2,3-epoxyhexanoyl-*S*-ACP (FA-ACP) and Ser-*S*-PCP were performed (Fig. 2). Control reactions were conducted by omitting the C domain from the reaction mixture. After incubation, the

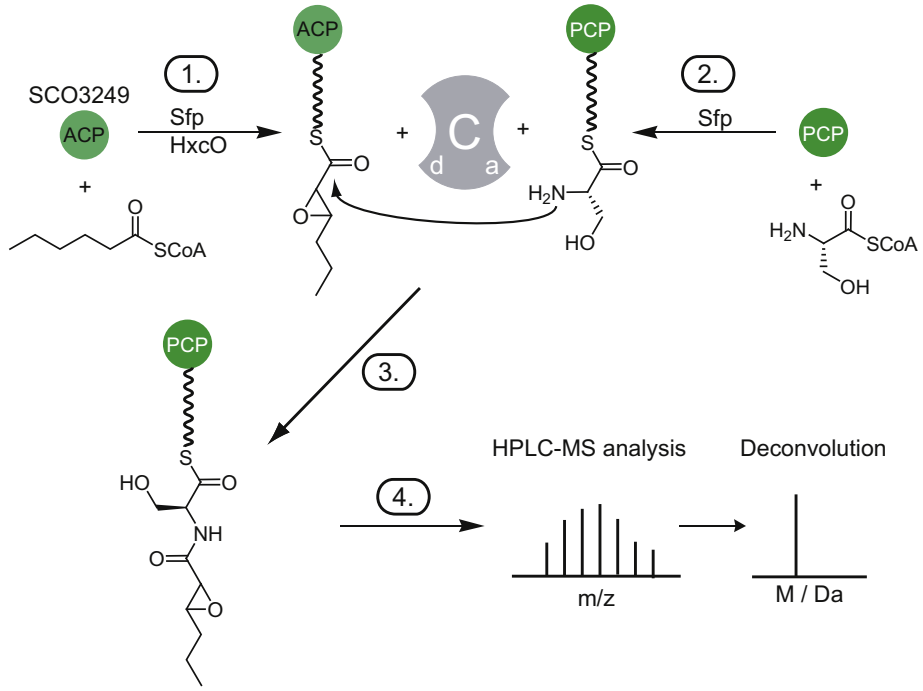

Fig. 2. Strategy for the investigation of the lipoinitiation in CDA biosynthesis. *Apo*-CPs are first loaded with the corresponding CoA thioesters by Sfp (1,2). Gel filtrated loaded CPs are then incubated with the excised C domain Cda-C1 (3) and analyzed by mass spectrometry (4). Putative catalytic sites within the C domain: d: donor site; a: acceptor site.

reaction mixtures were analyzed via HPLC-ESI-MS. Protein mass reconstruction was accomplished using ProMass Deconvolution for Xcalibur version 2.1. In the control reaction, masses of FA-ACP, Ser-PCP and of the respective *holo*-CPs could be identified (Fig. 3A, blue trace). In the reaction mixture containing the excised C domain, new signals at retention times of 16.5 and 26.8 min were detected, corresponding to the masses of the C domain and the expected condensation product 2,3-epoxyhexanoyl-serinoyl-*S*-PCP, respectively (Fig. 3A, red trace and Fig. 3B). Additionally, the peak area at 12.7 min is significantly increased, due to the accumulation of *holo*-ACP, which is also a product of the transfer reaction. The signal for the FA-ACP at 15.2 min decreases after incubation with the C domain in accordance with the fact that the fatty acid moiety is transferred onto the PCP-bound serine.

Previous biochemical and structural studies on C domains revealed the highly conserved HHxxxDG motif located in the active site of the protein that is associated with peptide bond formation [10–14]. Mutational studies of the second histidine of this motif in several C domains led to the loss of amide bond formation activity [8,12,15]. Therefore, we generated the Cda-C1 H156A and H157A variants by site-directed mutagenesis to test them in the condensation reaction. The Cda-C1 H157A variant resulted in a soluble protein that was unable to catalyze the condensation reaction to generate 2,3-epoxyhexanoyl-serinoyl-*S*-PCP (Fig. S4). Unfortunately, the variant H156A generated unsoluble protein and therefore was not tested.

To further characterize the C domain, substrate specificity at its donor and acceptor site was investigated using several substrates (Table 1). At the donor site of the C domain, only ACP-bound 2,3-epoxyhexanoic acid and to a certain extent also 2,3-epoxyoctanoic acid were accepted as electrophiles for the acylation of Ser-*S*-PCP. The stereoconformation of the epoxy group had no strong effect on the transfer reaction. We observed slightly more product formation when (2*R*,3*S*)-epoxyhexanoyl-ACP was used which is the product of epoxidation with HxcO, while HcmO gives the (2*S*,3*R*)-configured product. None of the other tested fatty acids, which differed in length from four to ten carbon atoms and the presence or absence of epoxidation, were valid substrates (Table 1). To elucidate the specificity of ACP/C domain interaction at the donor site, the ACP was substituted by DptF, an ACP from daptomycin biosynthesis [16], and AcpK, which is involved in polyketide biosynthesis in *Bacillus subtilis* [17]. In both cases, loading with hexanoic acid and epoxidation by HxcO and HcmO could be achieved (Table S1), but transfer of 2,3-epoxyhexanoic acid onto serine was not observed.

The acceptor site showed a considerably more relaxed specificity with regard to the carrier protein used. Both tested PCP domains, the initial PCP domains from SrfAA and DptA, could be loaded with serine and were acylated with the fatty acid moiety in the presence of Cda-C1. However, the amino acid tethered to the PCP domain could not be arbitrarily substituted. Beside serine, only threonine was accepted for acylation to a certain extent. Acylation of the other tested amino acids (lysine, asparagine or glutamate) could not be observed (Table 1).

4. Discussion

Lipopeptides feature a fatty acid moiety linked to the N-terminus of the peptide chain and all NRPS catalyzing this reaction contain an N-terminal C domain that is putatively assigned for the selection and incorporation of the fatty acid into the peptide chain. This step being the first in natural product assembly is thus referred to as lipoinitiation.

In this study we explore the specificity and the mechanism of the lipoinitiation reaction of the peptide assembly line in CDA biosynthesis, by using the dissected starter C domain Cda-C1 to characterize the *N*-acylation of the N-terminal amino acid serine. The biosynthesis of the fatty acid moiety, 2,3-epoxyhexanoic acid, occurs directly on the ACP SCO3249 through gene products of the *fab* genes located in the CDA biosynthetic gene cluster and probably by enzymes from the primary metabolism [4,9]. This study proves that 2,3-epoxyhexanoyl-ACP is directly used as a substrate by

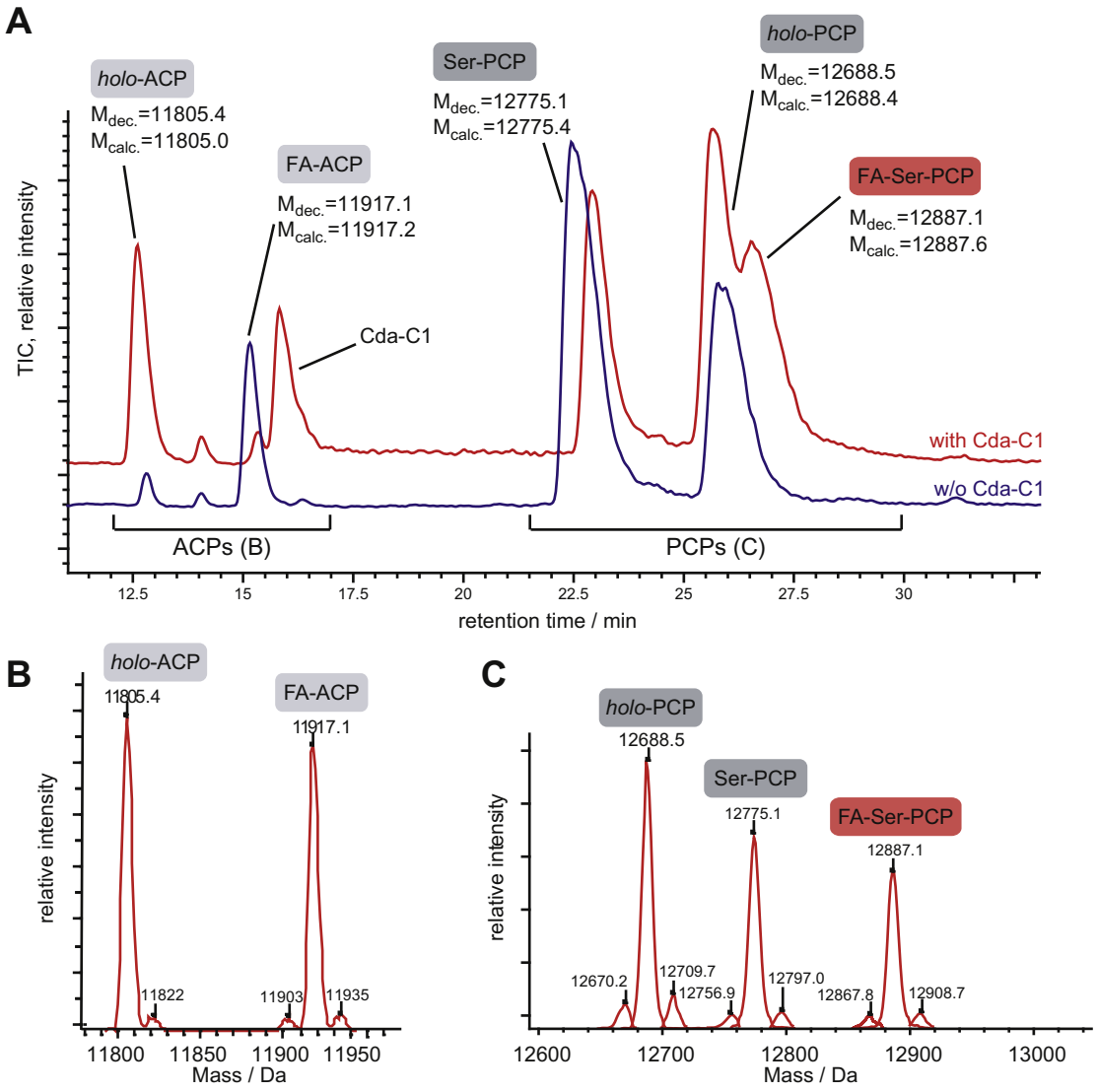

Fig. 3. *N*-Acylation of PCP-tethered serine by Cda-C1. Reaction products were analyzed by HPLC-MS. (A) HPLC-chromatograms of the control reaction without C domain (blue trace) and the lipoinitiation reaction with C domain (red trace). Peaks are labeled with the corresponding protein, whereas ACPs are shown in light grey, PCPs in dark grey and the product 2,3-epoxyhexanoyl-serinoyl-*S*-PCP (FA-Ser-PCP) in red. Experimental deconvoluted masses ($M_{dec.}$) and calculated masses ($M_{calc.}$) are given for each CP. (B) Deconvoluted ACP (12–17 min, B) and PCP (21.5–30 min, C) mass spectra of the reaction containing the excised C domain Cda-C1.

Cda-C1 for the transfer onto the peptide chain without further transthiolation. The utilization of the fatty acid as a carrier protein bound intermediate in the *N*-acylation reaction of CDA describes a novel mechanism for the lipoinitiation in NRP biosynthesis. In the surfactin system, it has been shown that the fatty acid moiety is activated *in trans* as a CoA thioester by fatty acyl CoA ligases from the primary metabolism and then directly incorporated into the peptide [8]. In the case of CDA, several genes in the biosynthetic cluster are needed for fatty acid biosynthesis and activation whose homologues are missing in the biosynthetic gene cluster of surfactin.

The interaction between the ACP encoded in the CDA gene cluster and the starter C domain seems to be highly specific as this CP could not be replaced by other ACPs such as DptF or AcpK [16,17]. It has been shown in several cases that residues belonging to helix-II, loop2 and helixIII of different CPs are involved in specific protein-protein interactions [18,19]. For NRPSs it was shown that the communication between different synthetases occurs via specific residues located in the linker regions defined as the com-motifs [20]. If similar interaction mechanisms can be found between Cda-C1 and ACP remains to be clarified. The generally high specificity of the C domain for the incoming CP-bound substrates both at the acceptor and the donor site assures a correct assembly of the natural product [1,5,6]. Especially for proteins acting *in trans*, as it is the case during lipoinitiation of CDA biosynthesis, a specific interaction is important to allow the incorporation of the correct building blocks. This may explain the high specificity of the starter C domain at its donor site for the acyl-ACP. In contrast, Cda-C1 shows a relaxed specificity for the CP at the acceptor site but high selectivity for the attached substrate. This makes the C domain a challenging target for domain swapping to generate new lipopeptides.

In vivo studies in *Streptomyces coelicolor* showed that alteration or deletion of enzymes synthesizing the fatty acid moiety leads to new variants of CDA that points towards a somehow relaxed specificity during this lipoinitiation reaction [21,22]. The deletion of *hxcO* leads to the production of a hexanoyl-CDA variant, which lacks the epoxide group found in wild-type CDA [22]. In contrast, the transfer of the hexanoyl residue was not observed in our in vitro studies. The accumulation of hexanoyl-*S*-ACP in the deletion mutant strain could shift the equilibrium towards the products and would therefore lead

Table 1
Substrate specificity of the C domain Cda-C1 for the *N*-acylation of aminoacyl-PCP.

	Substrate at donor site	$M_{exp.}$ (acyl-Ser-PCP)[a]/Da	$M_{calc.}$ (acyl-Ser-PCP)[a]/Da
Fatty acids	Butanoic acid	–	12758.4
	Epoxybutanoic acid	–	12772.4
	Hexanoic acid	–	12871.4
	2,3-Epoxyhexanoic acid	12887.1	12887.6
	Octanoic acid	–	12899.4
	2,3-Epoxyoctanoic acid	12914.6	12915.6
	Decanoic acid	–	12927.4
ACPs	DptF	–	12887.6
	AcpK	–	12887.6
	Substrate at acceptor site	$M_{exp.}$ (epoxyhexanoyl-aa-PCP)[a]/Da	$M_{calc.}$ (epoxyhexanoyl-aa-PCP)[a]/Da
Amino acids	Ser	12887.1	12887.6
	Thr	12900.4	12901.6
	Lys	–	12928.2
	Asn	–	12914.6
	Glu	–	12929.6
PCPs	SrfAA-PCP1	11339.0	11338.2
	DptA-PCP1	12887.1	12887.6

Reaction products which were not detected are indicated with dashes (−).
[a] In studies where the donor site and the amino acid at the acceptor site were varied DptA-PCP1 was used as the CP at the acceptor site.

to the saturated fatty acid in the CDA derivative. The same could be true for the observed incorporation of butanoic and 2,3-epoxybutanoic acid in vivo through modification of the FabF3 specificity [21], which was not observed in our in vitro study using the excised starter C domain.

The lipoinitiation in CDA biosynthesis describes a new third distinct mechanism for this reaction beside those observed in the NRP/PK hybrid mycosubtilin and the pure NRP surfactin systems [7,8]. The mechanisms of lipoinitiation in surfactin and CDA biosynthesis are quite similar, however differring in the mode of fatty acid activation. The starter C domain of the surfactin synthetase selects a CoA-activated fatty acid, whereas the C domain Cda-C1 uses 2,3-epoxyhexanoic acid tethered to an ACP, which is probably recognized by additional protein–protein interaction. The biosynthetic gene cluster of daptomycin, a related lipopeptide antibiotic that is in clinical use since 2003 [23], also contains a dedicated ACP and a starter C domain with high homology to Cda-C1 (39% identity, 52% similarity) pointing towards a more widespread utilization of this lipoinitiation mechanism [24].

All known classes of C domains associated with amide bond formation share a common catalytic core motif HHxxxDG, including starter C domains like Cda-C1 [11]. The substitution of the second histidine (H157) in Cda-C1 by alanine led to an inactive protein. This is in agreement with previous biochemical and structural studies of C domains in which the second histidine of this core motif was shown to be essential for the catalytic activity. This points towards a common mechanism of amide bond formation in C domain catalyzed reactions during elongation and initiation [8,12,15].

In conclusion, this study provides detailed insight into a new mechanism of lipoinitiation in acidic lipopeptide biosynthesis that broaden our understanding of lipopeptide biosynthesis by defining substrates involved and selectivities in the lipoinitiation reaction of CDA biosynthesis. This knowledge is necessary for the further development of drug design strategies employing techniques like domain swapping or alteration of enzymes involved in the synthesis of the fatty acid moieties found in lipopeptides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.003.

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